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Kinetics of migration of colloidal particles in meat muscles in the absence and presence of a proteolytic enzyme to simulate non-motile bacteria penetration

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ABSTRACT

In this study, migration rate and spatial distribution of colloidal particles with and without proteolytic enzymes were studied after injection in pork loin muscle cubes for 5 days at 25 °C to simulate behavior of non-motile bacteria. Samples were monitored daily by light microscopy and confocal laser scanning microscopy and particle distribution and movement analyzed by image analysis (MATLAB®). The extent of proteolytic activity was assessed by determining the tyrosine content in muscle tissue. Results showed that particles diffused from the injection hot spot into the inner structure of the meat ($1.35 \pm 0.99 \times 10^{-12}$ to $2.39 \pm 2.02 \times 10^{-12}$ m^2/s). Upon addition of protease, the migration rate increased, i.e. the particle distribution became more widespread due to proteolysis of meat proteins reducing resistance to mass transfer. Results indicate that particles are able to diffuse into the densely packed fiber structure of meat muscles, which is contrary to the long held belief that such penetration may not occur in the absence of extensive proteolysis or mechanical damage of tissue.

1. Introduction

The presence or absence of microbes in meat products plays a key role in the production of high-quality and safe products. For many years, it has been considered that muscle fibers are too densely structured to allow for microorganisms to migrate into the interior of tissue (Elmossalami & Wassef, 1971; Gill & Penney, 1977). Instead, bacteria were thought to be mainly present on the surface. Such surface contaminations may increase after slaughtering because biological mechanisms preventing contaminations have ceased to function, and protective coverings have been removed (Elmossalami & Wassef, 1971). Some later studies though demonstrated that microorganisms appeared to be able to penetrate muscle tissue, and authors attributed this to a wide range of different mechanisms (Anderson, Marshall, & Dickson, 1991; Elmossalami & Wassef, 1971; Gill, Leet, & Penney, 1984; Gill & Penney, 1977; Gill & Penney, 1982; Gill, Uttaro, Badoni, & Zawadski, 2008; Gupta & Nagamohini, 1992; Sikes & Maxcy, 1980; Thomas, O'Rourke, & McMeekin, 1987; Warsow, Orta-Ramirez, Marks, Ryser, & Booren, 2008).

The fact that some of the early studies had shown that only specific pathogenic bacteria were able to penetrate muscle tissue (Elmossalami & Wassef, 1971), led other authors to conclude that proteolytic activity was one of the key requirements to allow bacteria to colonize the interior of muscle systems by breaking down the connective tissue between muscle fibers (Gill & Penney, 1977; Gill & Penney, 1982; Gupta & Nagamohini, 1992). Authors postulated that this was the reason that non-proteolytic bacteria could not colonize the interior of muscle systems. Moreover, these studies showed that penetration only occurred during bacterial growth, and was accomplished by both motile and non-motile bacteria alike (Gill & Penney, 1977; Gill & Penney, 1982; Gupta & Nagamohini, 1992). However, these results were in contradiction to studies on chicken muscle tissue, where only motile bacteria were detected in the inner structure (Thomas et al., 1987). Furthermore, bacterial migration was found to be dependent on a great number of intrinsic and extrinsic factors, such as fiber orientation, hydration of meat proteins and the availability of pores and canals in the muscle tissue (Elmossalami & Wassef, 1971; Sikes & Maxcy, 1980; Thomas et al., 1987). Thus, if one reviews food science literature, it becomes apparent that there are many open questions regarding the distribution, location and pathways of bacteria in structured solid food, and in particular in muscle systems (Jongenburger, Bassett, Jackson, Zwietering, & Jewell, 2012; Wilson et al., 2002).

One approach to learn more about the behavior of bacteria in muscle tissue is to use a model system composed of colloidal particles combined with defined enzymatic treatments, and observe their behavior

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in meat tissue. Such a model system allows one to overcome the problem of the simultaneous occurrence of motility and proteolytic activity of microorganisms which makes a differentiation of effects difficult. For example, the approach has been successfully used to assess the efficiency of removal of microorganisms from carcasses during washing (Anderson et al., 1991; De Zuniga, Anderson, Marshall, & Iannotti, 1991). However no detailed examination of the development of a three-dimensional spatial distribution of bacteria was done.

A useful method to detect the location and pathways of bacterial penetration in food systems is by using microscopic imaging techniques. For example, light microscopy (LM) was used to investigate the potential for bacteria to penetrate muscle fibers undergoing extensive shrinkage during rigor (Gill et al., 1984). With the advent of confocal laser scanning microscopy (CLSM), this technique has been recommended to visualize the presence of bacteria in the muscle tissue. An early study by Delaquis and coauthors demonstrated that CLSM allowed the process of bacterial meat spoilage to be observed using an acridine orange stain (Delaquis, Gariépy, & Montpetit, 1992). A combination of staining and image analysis has shown to be useful to conduct not just qualitative but also quantitative analysis of such processes (Nunan et al., 2001; Ronneberger et al., 2008; Schönholzer, Hahn, Zarda, & Zeyer, 2002). This is in difference to earlier reports on bacterial distributions in muscle systems using microscopic images (Delaquis et al., 1992; Gill & Penney, 1977; Gill & Penney, 1982; Gill et al., 1984; Thomas et al., 1987).

The aim of this study was to use this recently developed quantitative analysis by microscopy to characterize the time-dependent development of a spatial distribution of stained colloidal particles injected into an intact meat structure to calculate apparent diffusion coefficients in the presence and absence of proteolytic enzymes. We postulated that by combining light microscopy and CLSM techniques, we might be able to obtain insights into the ability of especially non-motile proteolytic or non-proteolytic bacteria to penetrate muscle tissue. Previous studies assessed the penetration rate of microorganisms in meat with cell counting methods after incubation as a function of incubation time and sample length (Elmossalami & Wassef, 1971; Gill & Penney, 1977; Gupta & Nagamohini, 1992; Sikes & Maxcy, 1980; Thomas et al., 1987; Warsow et al., 2008). This yields averages but no detailed insights about the three-dimensional location pattern that develops over time. In contrast light microscopy combined with CLSM may provide a more detailed picture allowing conclusions about the underlying physical processes that drive the penetration of particulates. This fundamental understanding is also required to later use bacteria as starter cultures in whole muscle systems such as raw fermented hams with an injection process, since their ability to colonize the system is crucial to them improving color and aroma of such products. This study was therefore conducted as a first step towards an industrial use of starter cultures to manufacture raw fermented hams.

2. Materials and methods

2.1. Preparation of meat samples

Fresh pork from *Musculus longissimus dorsi* was obtained from a local central market (Mega, Stuttgart, Germany). A whole muscle was used for each trial and the muscle fascia was removed prior to use. Meat cubes (edge length of 30 mm) were prepared for the microscopy investigations and, additionally, some meat was minced to 3 mm using a meat grinder (Seydelmann, Stuttgart, Germany) for later use in the enzyme activity studies (see below). Two different experiments were carried out: one sample group was treated with particles mixed with defined doses of a proteolytic enzyme and one sample group did not contain the enzyme (control). All experiments were carried out in duplicate. The commercial enzyme solution Neutrase® 0.8 L (Novozymes, Bagsvaerd, Denmark; EC No. 3.4.24.28; CAS registry number: 9080-56-2) is a metallo protease produced by *Bacillus amyloliquefaciens* with a declared activity of 0.8 AU-N/g based on the manufacturer's specification (approx. 4% (w/w) neutral protease). Neutrase® has a high activity at neutral pH (pH 5–7) and a medium level activity at temperatures around 20–30 °C. It was hence selected for use in raw meat due to the intrinsic and extrinsic conditions chosen (meat pH: 5.5–6.5 (Ranken, 2000), storage temperature: 25 °C).

2.2. Light microscopy

2.2.1. Sample preparation

Blue-dyed polystyrene particles (diameter: 2.17 μm ± 0.04 μm) in 5% (w/v) aqueous suspension were obtained from microParticles GmbH (PS-FB-L1255, Berlin, Germany) and were used. They were selected because their particle size is similar to that of cocci bacteria used as starter cultures. For example *Staphylococcus carnosus* cells have particle sizes ranging from 0.5 to 1.5 μm diameter (Schleifer & Fischer, 1982). Moreover, they have a narrow size distribution and a high contrast in the meat tissue. The injection process of particles in meat cubes was used to simulate an injection of starter cultures in raw fermented ham. Injection solution for enzyme treatment contained 10^{-3} AU-N/g meat Neutrase® (Zapelena, Ansorena, Zalacain, Astiasaran, & Bello, 1998) and 0.625% (w/v) blue particles. The solution for control samples contained only 0.625% (w/v) particles. Each meat cube (21.1 g ± 1.1 g) was weighed (Sartorius MC1, Göttingen, Germany) and 40 μL injection solution was injected into the center of the cube (Fig. 1a) using a syringe (50 μL glass syringe, HP). Injected meat cubes were immediately labeled and packaged in plastic wrap to avoid humidity losses. All cubes were incubated at 25 °C and 75% rel. humidity in a climatic chamber (Ness, Remshalden, Germany) for a maximum of 5 days with daily sampling. On sampling day, each cube was cut in 27 smaller cubes of 10 mm edge length – henceforth referred to as specimens – using a scalpel. Seven specimens around the injection center were selected to be used for further investigations (Fig. 1b). Each of the specimens was put into a 7 mL vial with 4% paraformaldehyde (PFA; Carl Roth GmbH, Karlsruhe, Germany) in phosphate-buffered saline (PBS) over night (approx. 12 h) for fixation (Mulisch & Welsch, 2010). Afterwards, each specimen was washed three times with PBS for 15 min while shaking and stored in 15% sucrose (Carl Roth GmbH, Karlsruhe, Germany) in PBS. Prior to cryo-cutting, SuperFrost® microscope slides (Carl Roth GmbH, Karlsruhe, Germany) were covered with poly-L-lysine (Sigma Aldrich, Steinheim, Germany) (Mulisch & Welsch, 2010). Formaldehyde-fixed specimens were mounted onto a specimen holder (Tissue-Tek® O.C.T.™, Sakura Finetek, Japan) and cryo-fixed by rapidly plunging them into liquid nitrogen. Specimens were sectioned in three planes with a distance q of approximately 250 μm to 8–10 μm thick sections (Fig. 1b) using a cryostat (slee cryostat mtc, Mainz, Germany) and collected on poly-L-lysine microscope slides. Sections were directly mounted and cover-slipped with Mowiol® 4–88 (Carl Roth GmbH, Karlsruhe, Germany).

2.2.2. Observation

Light microscopy was performed using an inverted Nikon Eclipse Ti microscope (Nikon, Düsseldorf, Germany). The samples were examined using object lenses of 4× (Nikon, Plan Fluor) and 60× with oil immersion (Nikon, Plan Apo VC). NIS Elements Br 3.2 Imaging Software (Nikon, Düsseldorf, Germany) was used for photo documentation and free large image stitching. Images of 1280 × 960 pixels were acquired using 4× objective magnification (scale β of 3.43 μm/pixel). The 60× objective recorded images with 2560 × 1920 pixels (scale β of 0.12 μm/pixel).

2.3. Confocal laser scanning microscopy

2.3.1. Sample preparation

Fluorescent green (FluoGreen) dyed polystyrene particles (diameter: 2.07 μm ± 0.03 μm; $\lambda_{Excitation}$: 502 nm; $\lambda_{Emission}$: 518 nm) in 2.5% (w/v) aqueous suspension were obtained from microParticles GmbH